ARTICLE

Loss of Concurrent Regulation of the Expression of BIF-1, BAX and Beclin-1 in Primary and Metastatic Melanoma

Running title: Dysregulated expression of BIF-1, BAX and Beclin-1 in melanoma

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Abstract

Melanoma is one of the most aggressive and drug-resistant cancers. Despite novel promising therapeutic strategies, the prognosis of metastatic melanoma patients remains poor and it is often associated with high relapse rates. Endophilin B1, also known as BIF-1, is a multifunctional protein involved in several biological processes such as autophagy and apoptosis. BIF-1 promotes apoptosis through binding to BAX and its translocation to the mitochondrial outer membrane. On the other hand, BIF-1 can interact with Beclin-1 through UVRAG to promote autophagy. Several reports suggest an ambiguous role of BIF-1 in cancer development and progression. For example, it has been demonstrated that the expression of BIF-1 is reduced in both primary and metastatic melanoma and that the reduction of BIF-1 expression is associated with reduced overall survival of melanoma patients. Here we show that the expression of Beclin-1 and the active form of BAX are also reduced in melanoma patients. However, while we observed strong positive correlations between the expression of BIF-1 and Beclin-1 as well as between BIF-1 and BAX in benign nevi, these correlations were lost in primary and metastatic melanoma cells. These data indicate a dysfunctionality in the proximal molecular mechanisms which regulate the expression of BIF-1, Beclin-1 and BAX in primary and metastatic melanoma.

Introduction

Despite new promising therapeutic approaches, such as immunotherapy or targeted therapy, the prognosis of metastatic melanoma patients remains poor and is often associated with high tumor relapse rates. Unfortunately, prognostic factors that are currently used in clinics are not sufficient to clearly identify high-risk melanoma patients. BIF-1, also known as endophilin B1 or BAX-interacting protein 1, is part of the endophilin family of proteins, which are cytoplasmic proteins involved in numerous biological processes, such as mitochondrial membrane dynamics, apoptosis, autophagy, synaptic vesicle retrieval as well as receptor tyrosine trafficking and signaling [1]. We have previously demonstrated that the expression of BIF-1 is reduced in primary and metastatic melanomas as compared to healthy tissues and that the reduced BIF-1 levels are associated with a less favorable clinical outcome [2].

BIF-1 is mostly found in the cytosol and a fraction of the protein also resides on intracellular membranous compartments, including the Golgi complex and mitochondria [3-6], where it was shown to be required for the maintenance of mitochondrial morphology and function [2, 4]. Since its discovery as an interacting partner of BAX, several reports focused on its role in apoptosis. Moreover, depletion of BIF-1 expression in HeLa cells delays the conformational change of BAX and BAK, cytochrome *c* release, and caspase-3 activation induced by various intrinsic death signals [3, 6]. Besides being involved in BAX activation, it was also shown that BIF-1 forms a complex with Beclin-1 through UVRAG and positively modulates the formation of autophagosomes [7]. The involvement of BIF-1 in autophagy and apoptosis led to several investigations of the role of BIF-1 in cancer. Low expression of BIF-1 was found, besides melanoma [2], in invasive urinary bladder and gallbladder cancers [8] as well as in colorectal adenocarcinoma [9]. In contrast, high expression was reported in hepatocellular carcinoma [10]. Moreover, loss of BIF-1 in melanoma cells led to increased

ATP production, metabolic acidification, and mitochondrial respiration which was associated with higher proliferation rates both *in vitro* and *in vivo* [2].

Because of the role of BIF-1 in apoptosis and autophagy, we decided to investigate the correlation between the expression of apoptotic or autophagic proteins and BIF-1 in benign nevi as compared to primary and metastatic melanoma tissues. We show that the expression of active form of BAX is reduced in metastatic melanoma patients. Additionally, we could demonstrate that the expression of an essential autophagic protein Beclin-1 is also reduced in metastatic melanoma patients as compared to benign nevi. Moreover, the positive correlation between BIF-1 and the active form of BAX, Beclin-1 or the autophagic marker LC3B was lost in primary and metastatic melanoma cells. These data suggest that the proximal events regulating the expression of BIF-1, Beclin-1 and BAX are dysregulated in primary and metastatic melanoma cells.

Materials and methods

Study design and patients

The Tissue Micro Array (TMA) was constructed by the Department of Pathology, Bern. The study was approved by the Ethics Committee of the Canton of Bern. This cohort study aimed at investigating the role of BIF-1, BAX, LC3B and Beclin-1 in the pathogenesis of cutaneous melanoma. TMA included archived tissue samples of consecutive 65 melanocytic nevi, 41 primary and 30 metastatic melanomas obtained between the years 2003 and 2015 from patients at the Department of Dermatology, Inselspital, Bern, Switzerland.

Immunohistochemistry

IHC was performed as previously described [2, 11, 12]. Briefly, paraffin-embedded tissue sections were deparaffinized and rehydrated with graded ethanol dilutions which was followed by the antigen retrieval. Immunohistochemical staining was performed with the Dako REAL Detection System, using the Alkaline Phosphatase/RED kit, which also provided the secondary antibodies, according to the manufacturer's instructions (Agilent Technologies, K5005). The following primary antibodies were used: monoclonal anti-endophilin B1/BIF-1 antibody (Novus Biologicals, NBP2-24733; 1:100), polyclonal rabbit anti-Beclin-1 antibody (Abgent, San Diego, CA, USA; 1:100), monoclonal anti–BAX (clone 6A7, Santa Cruz Biotechnology, Dallas, TX, USA; 1:100) and polyclonal rabbit anti-LC3B antibody (Abgent, 1:100). The intensity of staining was evaluated by QuPath software [2, 13] and is presented as the mean optical density (OD) or by Image Pro Plus software and presented as integrated optical density (IOD) [11, 12].

Statistical analysis

Data were presented as means ± SD using the Prism Software v6 (GraphPad, La Jolla, CA, US). Significant values were represented according to the following convention: $p \geq 0.05^{ns}$, $p \leq 0.05$*, $p \leq 0.01$**, $p \leq 0.001$***, and $p \leq 0.0001$****. The follow-up data on primary and metastatic melanoma patients were divided for analysis into two groups according to the median expression of active form of BAX or Beclin-1 in their tumors. The group with "High BAX or Beclin-1" included patients with BAX or Beclin-1 levels that were higher than the median value of the whole population. The group with "Low BAX or Beclin-1" included patients with BAX or Beclin-1 levels that were lower than the median value of the whole population. Overall survival (OS) was defined as the time from random assignment to death from any cause. Disease-free survival (DFS) was defined as the time from random assignment to disease reoccurrence or progression. It was analyzed with the log-rank test and plotted as Kaplan-Meier survival curves.

Results

Reduced active BAX expression in metastatic melanoma patients

Our previous findings suggested that BIF-1 is a tumor suppressor in melanoma because it limits tumor growth by inhibiting mitochondrial functions. Several studies demonstrated that BIF-1 enhances apoptosis through promoting the conformational changes of BAX and BAK and the translocation of BAX to the mitochondria [3, 6]. Apoptosis is a crucial cell death mechanism by which damaged or transformed cells, which could be potentially cancerous, are eliminated. We decided to first investigate the expression of the conformational active form of BAX across several stages of melanoma development using a custom made tissue microarray (TMA) [2]. TMA contained 64 benign nevi, 41 primary melanoma and 30 metastatic melanoma tissue samples. Immunohistochemistry analysis of the TMA revealed a step wise decrease in the expression of active BAX from benign nevi via primary melanoma to metastatic melanoma (Fig. 1a). The expression of active BAX was significantly decreased in metastatic melanoma (mean OD = 0.1116 ± 0.03897) as compared to benign nevi (mean OD = 0.1588 ± 0.05966). To our surprise, further stratification of patients according to their median BAX expression in groups with high (OD > 0.1344 for primary melanoma and OD > 0.1274 for metastatic melanomas) or low active BAX expression showed that the active form of BAX does not have an impact on the OS or DFS of melanoma patients (Fig. 1b).

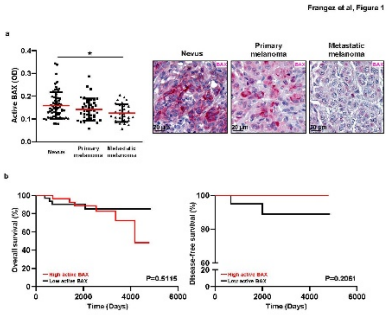

Fig. 1. Expression of BAX active monomeric form is reduced in metastatic melanomas compared with benign melanocytic nevi. a) Immunohistochemistry. Quantification of the BAX active monomeric form signal intensity. Intensity (mean optical density (OD)) values for individual patients are presented. The red lines represent the mean of all values. Statistical differences were analyzed by one-way ANOVA using a Kruskal-Wallis test and Dunn's *post hoc* test (left panel). Representative images of 65 benign nevi as well as 41 primary and 30 metastatic melanomas are shown (right panel). b) The same follow-up patients were divided into two groups ("high" and "low") on the basis of the median expression of BAX in their tumors. Kaplan-Meier curves for overall and disease-free survival are shown.

Reduced Beclin-1 expression in metastatic melanoma patients

In addition to apoptosis, BIF-1 has also been associated with autophagy. BIF-1 interact with Beclin-1 through binding to UVRAG and acts as a positive mediator of the class III PI(3) kinase and autophagy [7]. We next investigated the expression of Beclin-1 in benign nevi and melanoma patients. We observed a slight decrease in Beclin-1 expression in primary melanoma and a robust decrease of Beclin-1 expression in metastatic melanoma patients compared to benign nevi (Fig. 2a). Interestingly, stratification of patients according to their Beclin-1 median expression in groups with high (IOD > 9316.11 for primary melanoma and IOD > 808.272 for

metastatic melanomas) or low Beclin-1 expression revealed that Beclin-1 expression does not influence the OS or DFS of melanoma patients (Fig. 2b).

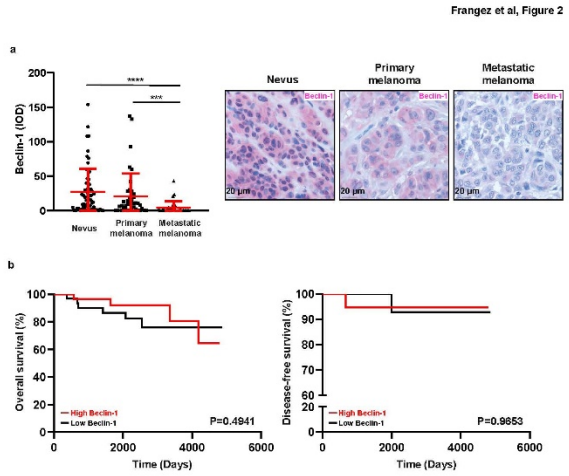

Fig. 2. Expression of Beclin-1 is reduced in metastatic melanomas compared with benign melanocytic nevi. a) Immunohistochemistry. Quantification of the Beclin-1 signal intensity. Intensity (integrated optical density (IOD)) values for individual patients are presented. The red lines represent the mean of all values. Statistical differences were analyzed by one-way ANOVA using a Kruskal-Wallis test and Dunn's *post hoc* test (left panel). Representative images of 65 benign nevi as well as 41 primary and 30 metastatic melanomas are shown (right panel). b) The same follow-up patients were divided into two groups ("high" and "low") on the basis of the median expression of Beclin-1in their tumors. Kaplan-Meier curves for overall and disease-free survival are shown.

The correlations of BIF-1 with active BAX, Beclin-1 or LC3B expression are lost in primary and metastatic melanomas

Since BIF-1 has been implicated in autophagy and apoptosis and since those processes are crucial in preventing tumor development or progression, we decided to investigate whether

proteins involved in autophagy or apoptosis have a similar expression pattern as BIF-1 in benign nevi and melanoma patients. For this purpose, we analysed the correlation between BIF-1 and the active form of BAX and Beclin-1. We observed a strong correlation between BIF-1 and the active form of BAX in benign nevi (Fig. 3a), suggesting a similar or the same mechanism regulating these two proteins in melanocytes. Interestingly, the correlation between BIF-1 and the active form of BAX was lost in primary and metastatic melanoma tissues (Fig. 3b and 3c).

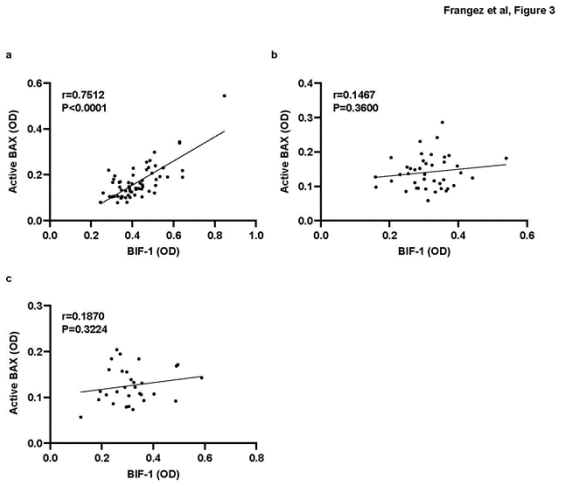

Fig. 3. BIF-1 positively correlates with BAX active monomeric form in benign nevi but not in primary or metastatic melanoma samples. (a-c) The scatter plot shows the correlation between the mean OD values of BAX active monomeric form with BIF-1 in benign nevi (a), primary melanoma (b) and metastatic melanoma (c) samples. The reported r and p -values show the Pearson correlation.

In addition, to the interaction with BAX, BIF-1 also forms a complex with Beclin-1 through UVRAG, influencing the induction of autophagy [7]. Beclin-1 is a central regulator of

autophagy and acts in the initiation phase of autophagy by forming the isolation membrane that engulfs the cytoplasmic material [14]. Therefore, we also investigated the expression of Beclin-1 and observed a strong correlation between active BAX and Beclin-1 in benign nevi (Fig. 4a) and loss of this correlation in primary and metastatic melanoma tissue (Fig. 4b and 4c).

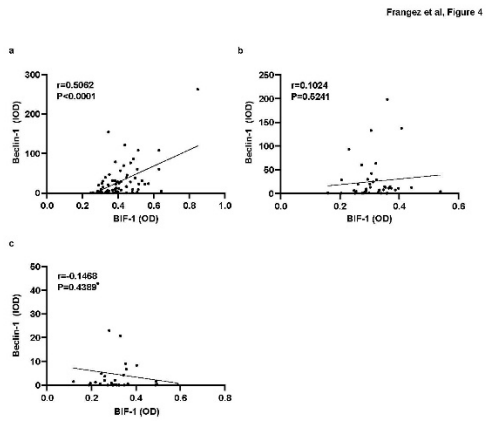

Fig. 4. BIF-1 positively correlates with Beclin-1 in benign nevi but not in primary or metastatic melanoma samples. (a-c) The scatter plot shows the correlation between the IOD values of Beclin-1 with mean OD values of BIF-1 in benign nevi (a), primary melanoma (b) and metastatic melanoma (c) samples. The reported r and p -values show the Pearson correlation.

Since BIF-1 is a positive regulator of autophagy, we also investigated the correlation between the expression of BIF-1 and a commonly used autophagic marker LC3B. Upon cleavage and lipidation with phosphatidylethanolamine (PE), LC3 is found on the inner and outer autophagosomal membrane [15]. In agreement with the role of BIF-1 in autophagy, we observed a strong positive correlation between the levels of BIF-1 and LC3B in benign nevi (Fig.5a) that was lost in primary and metastatic melanoma tissues (Fig. 5b and 5c). These

findings indicate that the proximal regulation of the expression of BIF-1, Beclin-1 and BAX is dysregulated in primary and metastatic melanoma cells.

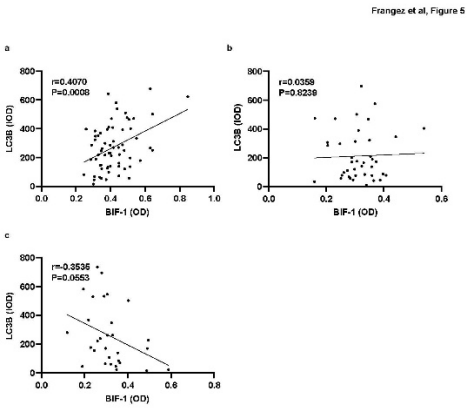

Fig. 5. BIF-1 positively correlates with LC3B in benign nevi but not in primary or metastatic melanoma samples. (a-c) The scatter plot shows the correlation between the IOD values of LC3B with mean OD values of BIF-1 in benign nevi (a), primary melanoma (b) and metastatic melanoma (c) samples. The reported r and p -values show the Pearson correlation.

Discussion

BIF-1 has been associated with several biological processes, in particular apoptosis, during which it interacts with BAX and promotes its conformational changes during cell death [3, 6]. We therefore wanted to investigate the BIF-1-BAX axis in our cohort of melanoma patients. Using a conformation-specific BAX antibody recognizing an activated form of BAX, we found a consistent reduction in active BAX associated with lower BIF-1 levels in metastatic melanoma tissue sections as compared to benign nevi. Moreover, we observed a positive correlation between the expression of BIF-1 and active BAX in nevi in which the mitochondrial apoptotic pathway is active [16]. In contrast, this correlation did no longer exist in melanoma

cells, suggesting that the apoptosis resistance, frequently arising in melanoma tumors, could be, at least partially, explained by downregulation of BIF-1.

Autophagy and apoptosis are both crucial cellular processes in determining the cellular fate and have a high impact on the development and progression of neoplasms [17]. Apoptosis is the most common physiological form of cell death in the absence of inflammation [18]. The intrinsic apoptotic pathway is regulated by the BCL-2 family of proteins and involves mitochondrial outer membrane permabilization (MOMP) which leads to the release of the pro-apoptotic factors such as cytochrome *c* and SMAC/DIABLO from the mitochondria to the cytosol. MOMP is followed by the activation of the caspase cascade which leads to cell death [19]. In healthy cells, the core pro-apoptotic regulators BAX and BAK shuttle between the outer mitochondrial membrane (OMM) and the cytosol [20, 21]. Under apoptotic conditions, BAX and BAK are activated and undergo dimerization and assemble at the OMM where they form multimeric pores [21]. The balance of interaction between pro-apoptotic and anti-apoptotic proteins, such as BCL-2 or BCL-XL, ensures appropriate apoptotic regulation in response to cellular stresses and other cell death triggering factors [19].

Loss of BAX expression has been reported as a negative prognostic marker in several cancers, such as breast, ovarian, pancreatic, and esophageal cancer [22-25]. Additionally, Fecker et al. have previously shown that loss of BAX in primary superficial-spreading melanomas was associated with tumor progression and reduced survival rates [26]. Moreover, downregulation of BAX in stage IIa melanomas was associated with an increased risk of development of metastasis and poor prognosis [27].

Interestingly, Beclin-1 has been identified as an interaction partner of BCL-2, BCL-XL and MCL-1. Those interactions may regulate the crosstalk between apoptotic and autophagic signalling pathways [17]. Besides being involved in BAX activation, BIF-1 also forms a complex with Beclin-1 through UVRAG and positively regulates the formation of

autophagosomes [7]. Here we show that the expression of Beclin-1 is reduced in melanoma tissues as compared to benign nevi. Moreover, besides the positive correlation between BIF-1 and BAX, we also observed a strong correlation between the expression of BIF-1 and Beclin-1 in melanocytes that was lost in primary and metastatic melanoma tissues. Furthermore, we observed the same correlation pattern between BIF-1 and the autophagic marker LC3B.

Several reports have pointed to a tumor suppressing role of autophagy. Beclin-1 has been described as a tumor suppressor gene because it was found to be monoallelically deleted in 40% to 75% of human breast, ovarian, and prostate tumors [28]. Moreover, allelic loss of Beclin-1 in mice led to high occurrence of tumors such as B cell lymphoma, hepatocellular carcinoma and lung adenocarcinoma [29]. A link between the role of Beclin-1 in melanoma was described in recently published work demonstrating that decreased Beclin-1 expression correlates with invasiveness and a decrease in 5-year survival after surgery [30].

In summary, our findings indicate that the proximal regulation responsible for a coordinated expression of BIF-1, Beclin-1 and BAX in melanocytes appears to be lost in primary and metastatic melanoma cells. It is likely that the reduced expression of BIF-1, Beclin-1 and BAX and its anticipated dysfunctional proximal regulatory mechanism contributes to tumorigenesis and drug resistance in melanoma.

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Acknowledgements

Ž.F. conceived, planned and performed the study, analyzed and interpreted data and wrote the paper. S.M.S.J. performed experiments; R.E.H. took clinical care of the melanoma patients; H.U.S. provided overall guidance, experimental advice and laboratory infrastructure and edited the paper; all authors read and approved the final manuscript.

Conflict of interests.

No potential conflict of interest was reported by the authors.

Compliance with Ethical Norms

All the procedures carried out in the research with participation of humans were in compliance with the ethical standards of the institutional and/or national ethics committee and with the Helsinki Declaration of 1964 and its subsequent changes or with comparable ethics standards. Informed voluntary consent was obtained from every participant of the study.

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Figure legends

Fig. 1. Expression of BAX active monomeric form is reduced in metastatic melanomas compared with benign melanocytic nevi. a) Immunohistochemistry. Quantification of the BAX active monomeric form signal intensity. Intensity (mean optical density (OD)) values for individual patients are presented. The red lines represent the mean of all values. Statistical differences were analyzed by one-way ANOVA using a Kruskal-Wallis test and Dunn's *post hoc* test (left panel). Representative images of 65 benign nevi as well as 41 primary and 30 metastatic melanomas are shown (right panel). b) The same follow-up patients were divided into two groups ("high" and "low") on the basis of the median expression of BAX in their tumors. Kaplan-Meier curves for overall and disease-free survival are shown.

Fig. 2. Expression of Beclin-1 is reduced in metastatic melanomas compared with benign melanocytic nevi. a) Immunohistochemistry. Quantification of the Beclin-1 signal intensity. Intensity (integrated optical density (IOD)) values for individual patients are presented. The red lines represent the mean of all values. Statistical differences were analyzed by one-way ANOVA using a Kruskal-Wallis test and Dunn's *post hoc* test (left panel). Representative images of 65 benign nevi as well as 41 primary and 30 metastatic melanomas are shown (right panel). b) The same follow-up patients were divided into two groups ("high" and "low") on the basis of the median expression of Beclin-1in their tumors. Kaplan-Meier curves for overall and disease-free survival are shown.

Fig. 3. BIF-1 positively correlates with BAX active monomeric form in benign nevi but not in primary or metastatic melanoma samples. (a-c) The scatter plot shows the correlation between the mean OD values of BAX active monomeric form with BIF-1 in benign nevi (a), primary melanoma (b) and metastatic melanoma (c) samples. The reported r and p -values show the Pearson correlation.

Fig. 4. BIF-1 positively correlates with Beclin-1 in benign nevi but not in primary or metastatic melanoma samples. (a-c) The scatter plot shows the correlation between the IOD values of Beclin-1 with mean OD values of BIF-1 in benign nevi (a), primary melanoma (b) and metastatic melanoma (c) samples. The reported r and p -values show the Pearson correlation.

Fig. 5. BIF-1 positively correlates with LC3B in benign nevi but not in primary or metastatic melanoma samples. (a-c) The scatter plot shows the correlation between the IOD values of LC3B with mean OD values of BIF-1 in benign nevi (a), primary melanoma (b) and metastatic melanoma (c) samples. The reported r and p -values show the Pearson correlation.